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(54) Title: LIPOXYGENASE

(57) Abstract: The invention provides sequence information of a microbial protein having lipoxy-genase activity and a method of producing the protein by recombinant DNA technology. More specifically, the inventors have isolated a gene encoding a lipoxygenase from Gaeu-mannomyces graminis, cloned it into an E. coli strain and sequenced it. A comparison shows less than 25 % identity to known lipoxygenase sequences, the closest being human 15S li-poxygenase. The inventors have expressed the lipoxygenase recombinantly and found that the recombinant lipoxygenase is glycosylated.

LIPOXYGENASE

FIELD OF THE INVENTION

The present invention relates to a polynucleotide encoding a lipoxygenase and its use for recombinant production of a lipoxygenase. The invention also relates to a method of obtaining a lipoxygenase by screening a DNA library with specific probes.

BACKGROUND OF THE INVENTION

Lipoxygenase is an enzyme that catalyzes the oxygenation of linoleic acid and produces a hydroperoxide. It is classified in Enzyme Nomenclature as EC 1.13.11.12. The enzyme is widely distributed in plants and animals. Encoding genes have been isolated from various sources, and the sequences have been published. Thus, GENESEQP W93832 and Genbank U78294 give the sequence of human 15S lipoxygenase.

Microbial lipoxygenases are known from a yeast *Saccharomyces cerevisiae*, a thermophilic actinomycete *Thermoactinomyces vulgaris*, from fungus *Fusarium oxysporum*, *Fusarium proliferatum* and *Gaeumannomyces graminis* (Su and Oliw, J. Biological Chemistry, 273 (21), 13072-13079 (1998)). No isolated gene encoding a microbial lipoxygenase has been described.

The prior art describes various uses of lipoxygenase, e.g. as a food additive to bread dough or noodles.

SUMMARY OF THE INVENTION

Here we for the first time provide sequence information of a microbial protein having lipoxygenase activity and a method of producing the protein in industrial scale. More specifically, the inventors have isolated a gene encoding a lipoxygenase from *Gaeumannomyces graminis*, cloned it into an *E. coli* strain and sequenced it. The genome of *G. graminis* contains approximately 60% of the G and C nucleotides, which made this work very difficult. A comparison shows less than 25 % identity to known lipoxygenase sequences, the closest being human 15S lipoxygenase. The inventors have expressed the lipoxygenase recombinantly.

Accordingly, the invention provides a polypeptide having lipoxygenase enzyme activity which:

a) has an amino acid sequence which has at least 50 % identity with the mature polypeptide of SEQ ID NO: 2 or 23;

b) is encoded by a nucleic acid sequence which hybridizes at 55°C with a complementary strand of the nucleic acid sequence encoding the mature polypeptide of SEQ ID NO: 1 or a subsequence thereof having at least 100 nucleotides;

c) has an amino acid sequence which can be obtained from the mature poly-peptide of SEQ ID NO: 2 or 23 by substitution, deletion, and/or insertion of one or more amino acids; or

d) is encoded by the lipoxygenase-encoding part of the DNA sequence cloned into a plasmid present in Escherichia coli deposit number DSM 13586.

The invention also provides a polynucleotide which comprises:

a) the partial DNA sequence encoding a mature lipoxygenase cloned into a plasmid present in Escherichia coli DSM 13586,

b) the partial DNA sequence encoding a mature lipoxygenase shown in SEQ ID NO: 2 or 23,

c) an analogue of the sequence defined in a) or b) which encodes a lipoxygenase and

i) has at least 50 % identity with said DNA sequence, or

ii) hybridizes at low stringency with a complementary strand of said DNA se-quence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

Other aspects of the invention provide a nucleic acid construct comprising the polynucleotide, a recombinant expression vector comprising the nucleic acid construct, and a recombinant host cell transformed with the nucleic acid construct. The invention also provides a recombinant method of producing the lipoxygenase, an oligonucleotide probe based on SEQ ID NO: 2 or 23 and a method of obtaining a lipoxygenase by screening a eukaryotic DNA library using the probe based on SEQ ID NO: 2.

Further, the invention provides a dough composition comprising a manganese lipoxygenase and a method for preparing a dough or a baked product made from dough, comprising adding a manganese lipoxygenase to the dough. The invention also provides a method of oxygenating a substrate selected from the group consisting of linolenic acid, arachidonic acid, linoleyl alcohol and a linoleic acid ester comprising contacting the substrate in the presence of oxygen with a manganese lipoxygenase. Finally, the invention provides a detergent composition comprising a manganese lipoxygenase and a surfactant.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

DNA encoding the lipoxygenase (LOX) may be derived from fungi, particularly *Ascomycota*, more particularly *Ascomycota incertae sedis* e.g. *Magnaporthaceae*, such as *Gaeumannomyces*, or anamorphic *Magnaporthaceae* such as *Pyricularia*, or alternatively anamorphic *Ascomycota* such as *Geotrichum*. An example is *G. graminis*, e.g. *G. graminis var. graminis*, *G. graminis var. avenae* or *G. graminis var.tritici*, particularly the strain *G.*

graminis var. graminis CBS 903.73, *G. graminis var. avenae* CBS 870.73 or *G. graminis var.tritici* CBS 905.73. The CBS strains are commercially available from Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

The inventors obtained two LOX-encoding DNA sequences from strains of *Gaeumannomyces graminis* and found that they have the sequences shown in SEQ ID NO: 1 and 22. They inserted a LOX-encoding gene into a strain of *Escherichia coli* and deposited it as *E. coli* DSM 13586 on 5 July 2000 under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE, Germany. The deposit was made by Novo Nordisk A/S and was later assigned to Novozymes A/S.

Lipoxygenase

The lipoxygenase of the invention is a manganese lipoxygenase, i.e. it has lipoxygenase activity (EC 1.13.11.12) with manganese in the prosthetic group. It is glycosylated and may have a molecular weight in the range 90-110 kDa, particularly 95-105 kDa. It is thermostable with a temperature optimum of 65-90°C, particularly 75-85°C. The lipoxygenase is stable against LAS (linear alkyl-benzene sulfonate) up to 400 ppm at pH 10. Mn-Lipoxygenase is enzymatically active between pH 5-12 with a broad optimum at pH 6-8.

A recombinant lipoxygenase may have a higher glycosylation and a higher thermostability. The recombinant lipoxygenase may have a molecular weight in the range 90-110 kDa, particularly 95-105 kDa. It may have a temperature optimum of 65-90°C, particularly 75-85°C.

Recombinant expression vector

The expression vector of the invention typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The lipoxygenase of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the lipoxygenase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism may be a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*. The production of the lipoxygenase in such host organisms may be

done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

Nucleotide probe

A nucleotide probe may be designed on the basis of the DNA sequence of SEQ ID NO: 1 or the polypeptide sequence of SEQ ID NO: 2, particularly the mature peptide part. The probe may be used in screening for LOX-encoding DNA as described below.

A synthetic oligonucleotide primer may be prepared by standard techniques (e,g, as described in Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) on the basis of the mature part of the amino acid sequence in SEQ ID NO: 2 or the corresponding part of the DNA sequence. It may be a degenerate probe and will typically contain at least 20 nucleotides.

Screening of eukaryotic DNA library

A polypeptide with lipoxygenase activity may be obtained by a method comprising:

a) preparing a eukaryotic DNA library,

b) screening the library to select DNA molecules which hybridize to the probe described above,

c) transforming host cells with the selected DNA molecules,

d) cultivating the transformed host cells to express polypeptides encoded by the DNA molecules, and

e) assaying the expressed polypeptides to select polypeptides having lipoxygenase activity.

The eukaryotic DNA library may be prepared by conventional methods. It may include genomic DNA or double-stranded cDNA derived from suitable sources such as those described above.

Molecular screening for DNA sequences may be carried out by polymerase chain reaction (PCR) followed by hybridization.

In accordance with well-known procedures, the PCR fragment generated in the molecular screening may be isolated and subcloned into a suitable vector. The PCR fragment may be used for screening DNA libraries by e.g. colony or plaque hybridization.

Hybridization

The hybridization is used to indicate that a given DNA sequence is analogous to a nucleotide probe corresponding to a DNA sequence of the invention. The hybridization may be done at low, medium or high stringency. One example of hybridization conditions is described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at approx. 45□C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55□C, particularly at least 60□C, more particularly at least 65□C, e.g. at least 70□C, or at least 75□C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using an x-ray film.

Alignment and identity

The nucleotide sequence of the invention may have an identity to the disclosed sequence of at least 75 % or at least 85 %, particularly at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of identity scores were done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Use of lipoxygenase

A manganese lipoxygenase such as that described above may be used in the following application, e.g. in analogy with the indicated publications.

The lipoxygenase can be used as an additive to dough for baked products such as bread, biscuits and cakes. Thus, the lipoxygenase can be used in a process for making bread, comprising adding the lipoxygenase to a dough, kneading the dough and baking the dough to make the baked product. SU 426640 A, JP 58190346 A[SLK1], JP 1165332 A[SLK2], JP 8322456,[SLK3] JP 10028516[SLK4], JP 08322456, JP 2964215. It can also be used in the preparation of noodles as described in JP 11299440 A.

The lipoxygenase may be used for bleaching, e.g. bleaching of beta-carotene, wheat flour or wheat dough. US 1,957,333 –1,957,337.

It can also be used for oxidizing mixtures of fatty acids to hydroperoxy fatty acids, as accelerators of lipid peroxidition, and as analytic tools to estimate linoleic and linolenic acids contents of certain oils.

The invention provides a detergent composition comprising the lipoxygenase and a surfactant, particularly an anionic surfactant such as LAS (linear alkyl-benzene sulfonate). Advantageously, the lipoxygenase has good stability in the presence of such surfactants. The detergent may be formulated as described in US 3635828 [SLK5]or US 5789362[SLK6]. The lipoxygenase can also be used to bleach stains from fabrics or hard surfaces as described in DK 9800352[SLK7]. Advantageously,

The lipoxygenase can be used for modification of starch as mentioned in JP 09163953, EP772980, JP 2000-106832. Also it can be used for protein modification as described in EP 947142, DE 19840069 or JP 61078361, or modification of oil (production of conjugated fatty acid) as mentioned in JP 5905128, US 3729379.

The lipoxygenase can be used for cross-linking a protein by oxidases, such as laccase, bilirubin oxidase etc. EP 947142.

The lipoxygenase can be used to obtain improved glutinousness and improved flavor of marine paste product such as Kamaboko, Hanpen, by adding lipoxygenase to fish meat. JP 61078361.

The lipoxygenase can be used to produce a process tomato product. It can be used for tomato pasta, salsa, ketchup and so on. EP 983725.

The lipoxygenase can be used for production of hydroperoxy fatty acid by reacting lipoxygenase with unsaturated 4-24C fatty acid. JP 11029410.

The hydroperoxides of linoleic acid or linolenic acid can be converted further to e.g. growth regulatory hormone jasmonic acid, and the product from arachidonic acid can be converted to physiological effectors leukotrienes and lipoxins.

Application of lipoxygenase should not be limited to the examples mentioned above. Since hydroperoxide, the product of lipoxygenase reaction, is good oxidant to create radical, lipoxygenase can be used for any other applications utilizing oxidation reaction, such as bleaching of food material or textile dyes, or polymerization of chemical compounds to produce plastic material or fiber.

Assay for lipoxygenase activity

The lipoxygenase activity was determined spectrophotometrically at 25°C by monitoring the formation of hydroperoxides. For the standard analysis, 10 μL enzyme was added to a 1 mL quartz cuvette containing 980 μL 25 mM phosphate buffer (pH 7.0) and 10 μL of substrate solution (10 mM linolenic acid dispersed with 0.2%(v/v) Tween20). The enzyme was typically diluted sufficiently to ensure a turn-over of maximally 10% of the added substrate within the first minute. The absorbance at 234 nm was followed and the rate was esti-

mated from the linear part of the curve. One unit causes an increase in absorbance at 234 nm of 0.001/min.

Determination of substrate specificity

The substrate specificity of the lipoxygenase was studied using the standard assays condition with a number of different compounds as substrate. All substrates were produced as dispersions with 0.2%(v/v) Tween20. The amount of compound added to make up these stock solutions was determined by mass, since viscosity made accurate measurement of volume impossible. The limiting rate constant and the specificity constant were determined by varying the amount of substrate added in the assays. The resulting rates were plotted against the concentration of substrate used. Finally, the plots were fitted by non-linear least squares regression to the theoretical hyperbolic curve of the Michaelis-Menten equation. The *cis-trans*-conjugated hydro(pero)xy fatty acids were assumed to have a molecular extinction coefficient of 23,000 $M^{-1}cm^{-1}$.

EXAMPLES

Materials and Methods

Molecular cloning techniques are described in Sambrook et al. (1989).

The following commercial plasmids and *E. coli* strains were used for sub-cloning and DNA library construction:

pT7Blue (Novagen)

pUC19 (TOYOBO, Japan)

E. coli JM109 (TOYOBO, Japan)

E. coli DH12□ (GIBCO BRL, Life Technologies, USA)

The following commercial Kits were used for cDNA cloning;

cDNA Synthesis Kit (Takara, Japan)

Marathon cDNA Amplification Kit (Clontech, USA)

Oligo dT cellulose powder (Invitrogen, Netherlands)

Labeling and detection of hybridization probe was done using DIG-labeling and detection Kit (Boehringer Manheim). Nylon membrane Hybond-N+ (Amersham, England) was used for DNA transfer for both southern blotting and colony hybridization.

Media and buffer solution

COVE-ar: per liter 342.3 g sucrose, 20 ml COVE salt solution, 10 mM acrylamide, 15 mM $CsCl_2$, 30 g Agar noble (Difco)

COVE2-ar: per liter 30 g sucrose, 20 ml COVE salt solution, 10 mM acrylamide, 30 g Agar noble (Difco)

COVE salt solution: per liter 26 g KCl, 26 g $MgSO_4$-$7H_2O$, 76 g KH_2PO_4, 50ml Cove trace metals.

Cove trace metals: per liter 0.04 g NaB_4O_7-$10H_2O$, 0.4 g $CuSO_4$-$5H_2O$, 1.2 g $FeSO_4$-$7H_2O$, 0.7 g $MnSO_4$-H_2O, 0.7 g Na_2MoO_2-$2H_2O$, 0.7 g $ZnSO_4$-$7H_2O$.

AMG trace metals: per liter 14.3 g $ZnSO_4$-$7H_2O$, 2.5 g $CuSO_4$-$5H_2O$, 0.5 g $NiCl_2$, 13.8 g $FeSO_4$, 8.5 g $MnSO_4$, 3.0 g citric acid.

YPG: per liter 4 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4$-$7H_2O$, 15 g glucose, pH 6.0.

STC: 0.8 M Sorbitol, 25 mM Tris pH 8, 25 mM $CaCl_2$.

STPC: 40% PEG4000 in STC buffer.

Cove top agarose: per liter 342.3 g sucrose, 20 ml COVE salt solution, 10 mM Acetamide, 10 g low melt agarose.

MS-9: per liter 30 g soybean powder, 20 g glycerol, pH 6.0.

MDU-2Bp: per liter 45 g maltose-$1H_2O$, 7 g yeast extract, 12 g KH_2PO_4, 1 g $MgSO_4$-$7H_2O$, 2 g K_2SO_4, 5 g Urea, 1 g NaCl, 0.5 ml AMG trace metal solution pH 5.0.

Materials.

alpha-^{32}P-dCTP (3000 Ci/mmol), dNTPs, alpha-^{33}P-ddNTPs, Hybond-N membranes, and DNA labelling beads (-dCTP), T-primed first-strand kit, and Thermo Sequenase kits were from Amersham Pharmacia Biotech (Uppsala, Sweden). TA cloning kits were from Invitrogen (Groningen, The Netherlands). *Taq* DNA polymerase and the enhanced avian RT-PCR kit were from Sigma (St. Louis, MO). Restriction enzymes were from New England BioLabs (Beverly, MA). *G. graminis* was obtained and grown as described by Su and Oliw (*supra*). Qiagen plant RNeasy mini and QIAquick gel extraction kits were from Merck Eurolab (Stockholm, Sweden). Degenerate primers for PCR were obtained from TIB Molbiol (Berlin, Germany), whereas sequencing primers were purchased from CyberGene (Huddinge, Sweden). 5´-RACE and reverse transcription of total RNA was performed with a kit (5´RACE system for rapid amplification of cDNA ends) from Life Technologies (Täby, Sweden).

Example 1: Determination of partial peptide sequences of LOX from *G. graminis*

A fungal strain of *Gaeumannomyces graminis var. tritici* was cultivated and lipoxygenase was recovered essentially as described in Chao Su and Ernst H. Oliw, J. Biological Chemistry, 273 (21), 13072-13079 (1998).

To obtain data from the N-terminal part of the enzyme, approximately 10 mg of enzyme was analyzed directly by using traditional edman degradation on the 494 Protein Sequencer, Applied Biosystems according to the manufacturer's instructions.

Another 40 microgram of sample was lyophilized down to around 20 µl and added 20 µl SDS-sample buffer containing DTT before incubation 30 min at 37°C and then boiling the sample for 3 min. 5 µl 0.5 M iodoacetamide in 1 M Tris-HCl, pH 7.5 was then added and

the sample was incubated 20 min at room temperature prior to running the sample on SDS-PAGE (4-20 %, Novex) according to the manufacturer's instructions. The gel was stained according to standard procedures from Novex.

The gelpiece (60 kDa) was subsequently cut out and minced with a blade. The gel pieces were washed 2X in 0.5 M tris pH 9.2/ACN (1:1) for 45 min at 37°C. The gel pieces were treated with 100% ACN for 10 min to introduce shrinking of the pieces. The ACN was removed and the pieces dries in speed-Vac. 200 ml 0.1 M NH4CO3 (AMBIC) was added and incubated for 15 min. AMBIC was removed and 100 ml ACN added. Again incubation for 10 min followed by removal of ACN and drying in speed-vac. The cycle with AMBIC was repeated 2X. After the last drying step 20ml 0.05 mg/ml trypsin in 0.1 M tris pH 9.2, 10% ACN was added. Incubation for 10 min. Then 300 ml 0.1 M tris pH 9.2, 10% ACN was added. Incubation was continued O.N. at 37°C. The supernatant was then removed (saved for control) and the peptides extracted from the gel by adding 30 ml 10 % TFA. After 5 min the TFA was withdrawn and collected. Further extraction was done 2 X by adding 150 ml 0.1% TFA, 60% ACN to the gel pieces and incubate for 30 min at 37°C. All extracts were collected (30ml+150ml+150 ml) and concentrated in the speed-vac to 50 ml. A sample of the concentrate (5 ml) was run on RP-HPLC on a Vydac C-18 column using solvent system of TFA/isopropanol to se if any peptides were present. The rest of the sample was run to collect the peptides. Controls with blank gel pieces were run in parallel. To minimize loss of peptide, selected fractions were sequenced directly without any repurification.

The resulting N-terminal sequence is shown as SEQ ID NO: 21, and two internal peptides (denoted fr 29 and 34) are shown as SEQ ID NOS: 19 and 20.

Further, around 100 µg lipoxygenase was added 40 µl 0.05 M potassium phosphate, 10 mM EDTA, 1% Triton X-100, 0.05% SDS, pH 7.3 and heated to 90°C for 4 min and allowed to cool. Then the sample was added 25 mU O-glycosidase (BSA free) and 800 mU EndoF glycosidase (Boehringer) and left over night at 37°C. The sample was then added 75 µl SDS sample buffer and run on SDS-PAGE (Novex 4-20%) in 7 lanes according to the manufacturer's instructions.

The 60 kDa bands were cut out from the gel minced and washed twice in eppendorf tubes with 400 µl of 0.5 M Tris-HCl, pH 9.2:ACN 1:1 for 45 min at 37°C. The gel pieces were then treated with 200 µl ACN for 10 min and then dried in the speed vac. 400 µl NH4HCO3 was added and left for 10 min before removing the supernatant and treating the pieces with another 200 µl of ACN for 10 min and then drying. 400 µl H2O was added and the sample left for 10 min before repeating the procedure with ACN again. The gel pieces was then added 25 µl 0.1 mg/ml trypsin + 300 µl 0.1 M Tris-HCl, 10% ACN, pH 9.2 and left over night at 37°C. After incubation 35 µl of 10 TFA was added and the supernatant were taken after 30 min for HPLC (Vydac C18, gradient to 80% acetonitril in 0.1 % TFA). The gel pieces were then further extracted twice with 150 µl 0.1 % TFA, 60 % acetonitril. The supernatant was

taken and evaporated in the speed vac to around 50 µl before adding further 100 µl 0.1% TFA and then re-evaporating down to 50 µl which was then run on the HPLC.

Three amino acid sequences (denoted fr 20, 21 and 25) were obtained, as shown in SEQ ID NOS: 16, 17 and 18.

Example 2: Cloning of genomic and cDNA clone of LOX from *G. graminis*

Preparation of fungal chromosomal DNA

A fungal strain *Gaeumannomyces graminis var. tritici* was cultivated in the YPG (composed per liter: 4 g Yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 15 g Glucose, pH 6.0) with gentle agitation at 25°C for 6 days. Mycelia was collected by filtration using Miracloth (*Calbiochem, USA*) and washed with deionized water twice. After briefly dried on paper filter, mycelia was frozen by liquid nitrogen and ground by motor on dry ice. Around 0.2g ground mycelia was put into a 1.5ml eppendorf tube and suspended in 0.5ml of buffer solution composed with 100mM NaCl, 25mM EDTA, 1% SDS and 50mM Tris-HCl (pH8). After addition of 3 micro-l of 25mg/ml proteinase K, the tube was incubated at 65°C for 30-60 minutes. The solution was extracted with the same volume of phenol and DNA was precipitated with 0.7 volume of isopropanol at –20°C. The pellet was re-suspended in 0.5ml of sterilized water and remaining RNA was digested by 50 micro-g of RNase at 37°C for 30 minutes. DNA was phenol extracted and ethanol precipitated again. The pellet was re-suspended in appropriate amount of sterilized water.

Preparation of mRNA and synthesis of cDNA

A fungal strain *Gaeumannomyces graminis var. tritici* was cultivated in the YPG with gentle agitation at 25°C for 6 days. After the lipoxygenase activity was confirmed, mycelia was collected and ground on dry ice as mentioned before to be used for the preparation of total RNA with phenol-chloroform method. Purification of mRNA from total RNA was performed with Oligo dT cellulose powder (Invitrogen, Netherland).

Synthesizing of cDNA was done with cDNA Synthesis Kit (Takara, Japan). The first strand cDNA was synthesized using 5-6 micro-g of heat denatured mRNA as the template in the mixture containing 1.0 mM each of dNTP, 4 µg of oligo$(dT)_{18}$ and 2 µg of random primer and 100 U of reverse transcriptase and 1st strand synthesis buffer. In total 50 µl of reaction mixture was kept at room temperature for 10 min, then incubated at 42ºC for 1 hour. After the incubation, the reaction mixture was chilled on ice for 2 min and subjected to 2nd strand cDNA synthesis. 1138 U of *E. coli* DNA polymerase and 5 µl of *E.coli* RNase H / *E. coli* DNA ligase mixture and 2nd DNA synthesis buffer was added to the 1st strand synthesis mixture and diluted up to 240 µl with DEPC-H_2O. The reaction mixture was incubated at 12ºC 1 hour, 22ºC 1 hour and 70ºC 10 min. Then 10 U of T4 DNA polymerase was added to the reaction

mixture and incubated at 37ºC 10 min. Synthesized cDNA was subjected to agarose gel electrophoresis to confirm the quality.

Isolation of a partial clone of LOX gene by PCR

The following primers were designed and synthesized based on the amino acid sequences determined in Example 1. The nucleotide sequence of linoleate diol synthase of *Gaeumannomyces graminis* (Genbank Accession #: AF124979) was used as a reference of codon usage.

Primer 1 for N-term side: SEQ ID NO: 9 (corresponding to amino acids 1-5 of N-terminal SEQ ID NO: 21).

Primer 2 for C-term side 1: SEQ ID NO: 10 (corresponding to amino acids 18-25 of fr 34, SEQ ID NO: 20).

Primer 3 for C-term side 2: SEQ ID NO: 11 (corresponding to amino acids 6-15 of fr 34, SEQ ID NO: 20).

Polymerase chain reaction (PCR) was employed using 0.6 µg of chromosomal DNA of *G.graminis* as the template in 50 micro-l reaction mixture containing 2.5 mM each of dNTP, 20 pmol each of primer 1 and 2, 2.5 units of LA taq polymerase (Takara, Japan) and GC buffer I supplied by Takara for LA taq. Reaction condition was shown below. LA taq polymerase was added to the reaction mixture after step 1.

Step	Temperature	Time
1	98 °C	10 mins
2	96 °C	20 sec
3	53 °C	45 sec
4	72 °C	(27 + 3 x cycle) sec
5	72 °C	10 mins

* Step 2 to Step 4 were repeated 50 times.

Second PCR reaction was employed in the reaction mixture described above but using 2 µl of first PCR product as template and primer 3 instead of primer 2. Reaction condition was the same as described above except step 2 to step 4 were repeated 30 times.

Amplified 1kb fragment was gel-purified using QIAquick™ Gel Extraction Kit (Qiagen) and subcloned into pT7Blue. Sequence of the PCR clone was determined as shown in SEQ ID NO: 3. From the deduced amino acid sequence of the PCR fragment, the primer 1 turned out to be hybridized to elsewhere than expected, however, amino acid sequence 250599Bfr25 (SEQ ID NO: 18) determined in Example 1 was found in continuous 216 amino acids sequence in the PCR fragment (SEQ ID NO: 8). Identity search showed that the 216 amino acid sequence had the highest identity to Human 15S Lipoxygenase (Genbank U78294,

GENESEQP W93832), Human arachidonate 12-Lipoxygenase (Swiss-Prot P18054) and *Plexaura homomalla* 8R-Lipoxygenase (GenBank AF003692, SPTREMBL O16025). The results indicated that the obtained PCR fragment contained lipoxygenase gene. The highest score of identity was obtained with Human 15S and was less than 25 %.

Cloning of genomic LOX gene

To obtain a full-length genomic clone, southern blotting was employed on genomic DNA of *G.graminis* using PCR fragment as a probe. Based on the result, genomic DNA was digested with *Sal*I and separated on 1.0% agarose gel. Around 6 kb of DNA digestion was recovered from the gel and ligated with BAP treated pUC19 lineared by *Sal*I. Ligation mixture was transformed into *E.coli* DH12S to construct a partial genomic library. It was screened by colony hybridization using the PCR fragment as probe, and a positive *E.coli* colony was isolated and the plasmid, termed pSG16, was recovered. The plasmid pSG16 contained a 6 kb SalI fragment from *G.graminis*. Out of 6kb of this fragment, sequence of 4.1 kb length including the PCR clone was determined as shown in SEQ ID NO: 4. The largest open reading frame (ORF) contained the above-mentioned 216 amino acid sequence as well as the similar sequences to fr 20, 21, 29 and 34, SEQ ID NOS: 16, 17, 19 and 20 but not the N-terminal sequence (SEQ ID NO: 21) determined in example 1. Two other small ORFs were found in the upstream of the largest ORF, but none of them had the N-terminal sequence neither. To find the right initial ATG codon, cDNA cloning was necessary.

Isolation of cDNA clone of LOX gene

Total RNA was extracted from the mycelia producing lipoxygenase and subjected for mRNA preparation by Oligo dT cellulose powder. The cDNA was synthesized from the mRNA using cDNA Synthesis Kit (Takara, Japan) and aiming to obtain full-length cDNA, 1-4kb of cDNA was gel-purified to be subjected for the construction of a partial cDNA library. Library was constructed by ligating with the adaptor of Marathon cDNA Amplification Kit (Clontech, USA), which allows the amplification of aimed cDNA with the Adaptor Primer (AP1) and a custom primer designed for the internal sequence of aimed clone.

For the amplification of cDNA of LOX, two primers, primer 4 (SEQ ID NO: 12) and primer 5 (SEQ ID NO: 13), were designed based on the sequence of genomic clone. C-terminal part was amplified with primer 4 and AP1, and N-terminal part was amplified with primer 5 and AP1.

PCR reaction mixture comprised of 2.5 mM dNTP, 30 pmol each of primer 4 and AP1 or primer 5 and AP1, 5 units of LA taq polymerase (Takara) and supplied GC buffer I. Reaction condition was shown below. LA taq polymerase was added to the reaction mixture after step 1.

Step	Temperature	Time
1	98°C	5mins
2	95°C	30sec
3	74°C	15sec
4	68°C	3mins
5	95°C	30sec
6	95°C	5mins
7	54°C	30sec
8	68°C	15sec

*Step 2 to Step 4 were repeated 15 times and the temperature of Step 3 was decreased 4°C after each 3 repeat. Step 6 to Step 8 were repeated 20 times.

As the results, 0.6kb and 1.6kb fragments were amplified for 5'-end and 3'-end respectively and the sequences were determined as shown in SEQ ID NO: 5 and SEQ ID NO: 6. Based on the sequence around the predicted initial ATG and stop codon TAA, the primer 6 (SEQ ID NO: 14) and primer 7 (SEQ ID NO: 15) were designed for the amplification of end-to-end cDNA. Also desired restriction enzyme sites were introduced at both ends for further plasmid construction.

Reaction mixture contained 0.08 µg of cDNA library, 2.5mM dNTP, 30 pmol each of primer 6 and 7, 1 units of LA taq polymerase (Takara) and GC buffer. Reaction condition was shown below. LA taq polymerase was added to the reaction mixture after step 1.

Step	Temperature	Time
1	98 °C	10 mins
2	96 °C	20 sec
3	53 °C	45 sec
4	72 °C	(27 + 3 x cycle) sec
5	72 °C	10 mins

* Step 2 to Step 4 were repeated 50 times.

PCR amplified 1.9 kb fragment was isolated and cloned into pT7Blue resulting in pSG26. Sequence of the full-length cDNA was determined. The deduced open reading frame consisted of 1857bp, which corresponded to 618 amino acids and a molecular mass of 67600 Da. Comparison with the genomic sequence turned out that the LOX gene contained one intron in the N-terminal side. Predicted N-terminal sequence by signal sequence determination program is "ALPLAAEDAAAT". Identity search with the full-length amino acid se-

quence showed that it had the highest identity to Human 15S Lipoxygenase (Genbank Accession number w93832), less than 25 %.

The plasmid pSG26 was transformed in *E. coli* JM109 and deposited at DSMZ as DSM 13586 with the accession date 5th July 2000.

Example 3: Expression of *G.graminis* LOX in *A. oryzae*

Host organism

Aspergillus oryzae BECh2 is described in Danish patent application PA 1999 01726. It is a mutant of JaL228 (described in WO98/123000), which is a mutant of IFO4177.

Transformation of *A. oryzae*

Aspergillus oryzae strain BECh2 was inoculated in 100 ml of YPG medium and incubated at 32°C for 16 hours with stirring at 80 rpm. Grown mycelia was collected by filtration followed by washing with 0.6 M KCl and re-suspended in 30 ml of 0.6 M KCl containing Glucanex® (Novo Nordisk) at the concentration of 30 µl/ml. The mixture was incubated at 32°C with the agitation at 60 rpm until protoplasts were formed. After filtration to remove the remained mycelia, protoplasts were collected by centrifugation and washed with STC buffer twice. The protoplasts were counted with a hematitometer and re-suspended in a solution of STC:STPC:DMSO (8:2:0.1) to a final concentration of 1.2×10^7 protoplasts/ml. About 4 µg of DNA was added to 100 µl of protoplast solution, mixed gently and incubated on ice for 30 minutes. 1 µl STPC buffer was added to the mixture and incubated at 37°C for another 30 minutes. After the addition of 10 ml of Cove top agarose pre-warmed at 50°C, the reaction mixture was poured onto COVE-ar agar plates. The plates were incubated at 32°C for 5 days.

SDS-PAGE

SDS polyacrylamide electrophoresis was carried out using the commercialized gel PAGEL AE6000 NPU-7.5L (7.5T%) with the apparatus AE-6400 (Atto, Japan) following the provided protocol. 15 µl of sample was suspended in 15 µl of 2x conc. of sample loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM Dithiothreitol, 4% SDS, 0.2% Bromophenol blue and 20% glycerol) and boiled for 5 minutes. 20 µl of sample solution was applied to a polyacrylamide gel, and subjected for electrophoresis in the running buffer (25 mM Tris, 0.1% SDS, 192 mM Glycine) at 20 mA per gel. Resulting gel was stained with Coomassie brilliant blue.

Construction of expression plasmid

The plasmid pSG26 containing cDNA of *G.graminis* LOX was digested by *Bgl*II and *Xho*I and 1.9 kb of fragment which contained the LOX gene was ligated with pMT2188 digested with *Bam*HI and *Xho*I. The plasmid pMT2188 has a modified *Aspergillus niger* neutral

amylase promoter, *Aspergillus nidulans* TPI leader sequence, *Aspergillus niger* glucoamylase terminator, *Aspergillus nidulans* amdS gene as a marker for fungal transformation and *S.cerevisiae* ura3 as the marker for *E.coli* transformation. Transformation was done with *E. coli* DB6507 in which pyrF gene is deficient and can be complemented with *S.cerevisiae* Ura3. Resulting plasmid was termed pSG27.

Expression of *G.graminis* LOX in *A. oryzae*

A. oryzae BECh2 was transformed with the plasmid pSG27 and selection positive transformants were isolated. Transformants were grown on COVE 2 -ar at 32ºC for 5 days and inoculated to 100 ml of MS-9 shaking flask. After the cultivation with vigorous agitation at 32°C for 1 day, 3 ml of each culture was transferred to 100 ml of MDU-2Bp in shaking flask to cultivate at 32°C for 3 days. Culture broth was centrifuged at 3500 rpm for 10 minutes and supernatant was collected. Lipoxygenase activities of the supernatant were determined spectrophotometrically as described before. Positive transformants showed about 50,000U/ml culture broth while untransformed *A. oryzae* BECh2 showed no activity. Culture supernatant was also subjected to SDS-PAGE analysis. Positive transformants showed 90-110 kDa smear band which indicated the protein was heavily glycosylated. Untransformed *A.oryzae* BECh2 did not show any major band.

Example 4: Purification of recombinant lipoxygenase

One gram of crude lyophilised lipoxygenase prepared as in the previous example was dissolved in 40 mL 25 mM Tris-HCl (pH 8.0) and then filtered (0.45 μm, type Millex-HV, Millipore). The above and subsequent steps were all carried out at room temperature. The filtrate was loaded on a SP-Sepharose Fast Flow (2.6 x 14 cm) with 25 mM Tris-HCl (pH 8.0) at 1 mL/min. The column was then washed with the same buffer at 2.5 mL/min until baseline was reached (approximately 4 column volumes). The bound protein was then eluted with a linear gradient from 0 to 330 mM NaCl in 25 mM Tris-HCl (pH 8.0) in 2 column volumes. Fractions of 10 mL were collected. The column was cleaned with 1 M NaCl in 25 mM Tris-HCl (pH 8.0). The fractions containing the majority of pure lipoxygenase, as estimated by SDS-PAGE and by activity assay, were pooled and concentrated using an Amicon cell (10,000 NMWL, YM10, Millipore). The enzyme was finally transferred into 50 mM sodium phosphate (pH 7.0) by dialysis and stored in aliquots at -20°C until use.

SDS-PAGE analysis showed that the lipoxygenase had been purified to homogeneity. The enzyme was found to have an estimated molecular weight of 90-110 kDa, somewhat higher than the theoretical value based on the amino acid sequence (65.6 kDa). This was taken as an indication of glycosylation. The protein was found to have a very high isoelectric point as demonstrated by the successful purification employing cation exchange chromatography.

Example 5: Determination of the gene and the deduced protein sequence of Mn-lipoxygenase

1. Amino acid sequences of internal peptides and the C-terminal amino acids of manganese lipoxygenase

Manganese lipoxygenase was purified to homogeneity as described by Su and Oliw (*supra*), using a strain of *G. graminis* (different from the previous examples). Internal peptides were generated, purified and sequenced by the Sanger method essentially as described for another protein of *G. graminis* (Hornsten L, Su C, Osbourn AE, Garosi P, Hellman U, Wernstedt C and Oliw EH, Cloning of linoleate diol synthase reveals homology with prostaglandin H synthases. *J Biol Chem* **274**(40): 28219-24, 1999). The N-terminal amino acid of Mn-lipoxygenase was blocked, but four C-terminal amino acid was obtained by C-terminal sequencing.

(i) C terminal amino acid sequence

These C-terminal amino acids were FLSV.

(ii) Internal amino acid sequences

The following eight internal amino acid sequences were obtained (where (K), (K/R) and (E) denotes the fact that Lys-C, trypsin and V8 cleaves peptides at the C-terminal side of K residues, K or R residues, and E residues, respectively):

(K)LYTPQPGRYAAACQGLFYLDARSNQFLPLAIK (amino acids 205-237 of SEQ ID NO: 23 with the substitution K206L)

(K/R)HPVMGVLNR (amino acids 295-304 of SEQ ID NO: 23 with Lys or Arg at position 295)

(K/R)LFLVDHSYQK (amino acids 196-205 of SEQ ID NO: 23 with Lys or Arg at position 196)

(E)M?AGRGFDGKGLSQG(W/M)PFV (amino acids 569-587 of SEQ ID NO: 23, except that amino acid 570 is uncertain Met and amino acid 584 is Trp or Met)

(K/R)GLVGEDSGPR (amino acids 365-375 of SEQ ID NO: 23 except that amino acid 365 was found to be Lys or Arg and 368 Val)

(K)TNVGADLTYTPLD/AD/WK/LP/ND/NE (amino acids 237-255 of SEQ ID NO: 23 except that amino acid 242 was found to be Ala, 250 Asp or Ala, 251 and Asp or Trp)

(K)G/F SGVLPLHPAw (amino acids 472-483 of SEQ ID NO: 23, except that amino acid 473 was found to be Gly or Phe, and amino acid 483 uncertain Trp)

(K) QTVDDAFAAPDLLAGNGPGRA (amino acids 532-553 of SEQ ID NO: 23 except that amino acid 536 was found to be Asp, and 552 Arg)

2. RT-PCR with degenerate primers generated cDNA of Mn-lipoxygenase

This part of the invention was difficult due to the high GC content of the genome of *G. graminis*.

Methods for isolation of total RNA from *G. graminis* and transcription of mRNA to cDNA had to be optimised. cDNA was often contaminated with genomic DNA in spite of digestion with DNAses and other precautions.

After considerable experimentation, using over 30 degenerate primers in various combinations, the first cDNA clone of Mn-lipoxygenase could be obtained by RT-PCR. It was obtained by the following degenerate primers, which were based on internal peptides 1 and 2 and above.

Mn60 (5´-AACCAGTTCCTSCCSCTCGCSATCAA)

Mn15R (5´-GTCGAGGTAGAAGAGGCCCTGRCAVGC),

EO3a (5´-CATCCSGTSATGGGYGTSCTBAA)

EOr3a (5´-CGGTTSAGGACRCCCATVACVGGRTG).

The primers Mn60 and EOr3A generated an RT-PCR band of about 230-bp and the primers EO3A and Mn15R generated an RT-PCR band of about 220-bp. A sense primer from this sequence (MnS2: 5´-CCGTTCAGCGTCGAGAGCAAGG) and an antisense primer from the other sequence (MnS1, 5´-TCTCGGGGATCGTGTGGAAGAGCA) amplified a fragment of 337-bp. The amplicon was sequenced and it contained the amino acid sequence of peptide1 in one of the reading frames. The amplicon was used as probe for Northern blot analysis and for screening of a genomic library (Hornsten et al., *supra*).

3. Screening of a genomic library of *G. graminis*

A genomic library of *G. graminis* in Lambda ZAP II was obtained as described by Bowyer P et al., *Science* **267**(5196): 371-4, 1995. It was screened with a probe of 0.33-kb from the cDNA sequence. Screening of over 100 000 plagues yielded 11 positive clones, which were plague purified by 2-3 additional rounds of phage screening. The Bluescript SK phagemid was excised with helper phage following published methods. Restriction enzyme analysis showed that all rescued phagemids contained the same insert of 8-kb.

4. Sequencing of the gene and coding region of Mn-LO of *G graminis*

Sequencing was performed of both strands using two different methods based on cycle sequencing. The sequencing was difficult due to the high GC content of the gene (over 60% GC).

3.4-kb of the genome of G. graminis was sequenced and the sequence of 2725 nucleotides of the Mn-lipoxygenase gene included an intron of 133-bp. The gene of Mn-lipoxygenase was identified by 5´-RACE from the starting point of transcription of 2mRNA, a^{1}gcaggttc, and the protein translation start point A^{72}TG (at nucleotide position 72). The C-terminal amino acids FLSV were found with the stop codon at position 2060-2062. Over 0.6-kb of the 3´-untranslated region was sequenced and tentative polyadenylation signals were found as shown below:

5-RACE and cDNA sequencing was used to confirm the deduced open reading frame and the exon-intron borders. The transcription start point, the translation start point and the translation end were determined as shown in SEQ ID NO: 22 and 23.

The Intron was found to have a length of 133 bp and to have the sequence shown as SEQ ID NO: 24. It was found to be located between nucleotides 372 and 373, i.e. between Ser108 and Arg109 of SEQ ID NO: 22.

Example 6: Expression of native and genetically modified Mn-lipoxygenase

We have subcloned a genomic segment (3-kb) containing the coding region of the Mn-lipoxygenase gene from the Bluescript SK phagemid into the multi cloning site (with *Spe*I and *Nsi*I sites) of the plasmid pGEM-5Zf (Promega) using the restriction enzymes *Spe*I and *Nsi*I.

The 5´-end and the intron were modified as follows. pGEM-5Z with the insert was cleaved with *Spe*I and *Bse*RI, which cut out the 5´-end of the gene and part of the genomic sequence with the intron (1323-bp). This piece was replaced in pGEM with a cDNA sequence of about 405-pb, which was obtained by cleavage of a PCR product of 448-bp with *Spe*I and *Bse*R1. This vector is designated pGEM_Met. The PCR product was generated with a sense primer specific to the translation start region (and with *Spe*I and *Nde*I site in the 5´-end of the primer, 5´-TTACTAGTCAT**ATG**CGCTCCAGGATCCTTGCT), and a gene specific antisense primer located at the 3´-end of the *Bse*R1 site. This cDNA part so inserted thus contained the beginning of the ORF (without the Intron positioned between nucleotides 372 and 373, between Ser108 and Arg109, as shown in the table above), so that the entire ORF was obtained in the vector pGEM_Met.

The 3´-end was modified with PCR, taking advantage of an *Bbv*CI site about 130-bp from the stop signal. The sense primer was gene-specific and located at the 5´-side of the restriction site, whereas the antisense primer was designed from the nucleotides of the terminal amino acids and contained, in addition, *Nde*I and *Nsi*I restriction sites. The pGEM_Met vector was cleaved with *Nsi*I and *Bbv*C1, and the excised fragment was replaced with the PCR product cleaved in the same way. This yielded the vector pGEM-Met_ter. The modified coding region of Mn-lipoxygenase in this vector can thus be excised with *Nde*I. All modifications have been confirmed by sequencing of the expression constructs.

1. Expression of Mn-lipoxygenase in procaryotic cells (*E. coli*)

The expression vector pET-19b has been linearized with *Nde*I, and the modified coding region of Mn-lipoxygenase has been excised with *Nde*I and ligated into this vector for expression in *E. coli*, as suggested by the manufacturer of the pET expression vectors (Stratagene). Studies of recombinant Mn-lipoxygenase expressed in *E. coli* is now in progress.

2. Expression of Mn-lipoxygenase in eukaryotic cells (*Pichia pastoris, Saccharomyces cerevisiae, Aspergillus nidulans, Gaeumannomyces graminis*)

We plan to use the Pichia Expression kit with the pCIC9 or related vectors (Invitrogen), which has to be slightly modified to fit our modified coding region of Mn-lipoxygenase. It is possible that glycosylation of recombinant Mn-lipoxygenase may differ between different hosts. We therefore plan to investigate a series of eukaryotic expression systems in *Saccharomyces cerevisiae, Aspergillus nidulans, Gaeumannomyces graminis.* Glucosylation may improve the stability of the recombinant enzyme.

3. Expression of Mn-lipoxygenase in eukaryotic cells (insect cells)

We plan to use the Drosophila Expression System (Schneider 2 cells) from Invitrogen using an expression vector without His tags at the C-terminal end.

4. Genetically modified Mn-lipoxygenase for expression.

Our discovery that Mn-lipoxygenase belongs to the lipoxygenase gene family opens large possibilities for rational modification of the structure. The 3D sequence of several lipoxygenases are known and Mn-lipoxygenase shows significant amino acid identity along many α-helices of soybean lipoxygenase-1 (Prigge ST, Boyington JC, Gaffney BJ and Amzel LM, Structure conservation in lipoxygenases: structural analysis of soybean lipoxygenase-1 and modeling of human lipoxygenases. *Proteins* **24**(3): 275-91, 1996), which has been used for modeling of many lipoxygenases. Both the metal ligands and other structurally important amino acids of Mn-lipoxygenase will be mutated in order to increase the bleaching properties and oxidative properties of the enzyme..

4.1 Site directed mutagenesis of amino acids of important alpha-helices.

Amino acid sequences of Mn-lipoxygenase align with α-helix 9 (Prigge et al., *supra*), which contains the WLLAK sequence and two His residues, which likely are Mn ligands. Systematic changes of amino acids in this helix might have profound effect on enzyme activity and bleaching properties. In the same way, an amino acid sequence of Mn-Lipoxygenase align with α-helix 18, which contain iron ligands and likely Mn-ligands (His and Asn). Other predicted α-helices of Mn-lipoxygenase, which should be mutated, correspond to α-helices 7, 8, 10-17, 19-22 of soybean lipoxygenase-1 (Prigge et al., *supra*). We predict that some of these genetically modified Mn-lipoxygenases may have totally different properties, and the bleaching effect may be enhanced. Predicted Mn ligands thus are 3 His residues, one Asp residue and one Val residue. Mn-lipoxygenase likely belongs to enzymes of the "2-His-1-carboxyl facial triad".

4.2 Site directed mutagenesis of amino acids of the C-terminal end.

We plan to mutate the terminal Val to an Ile or to other residues and to determine the bleaching properties of the mutated form.

4.3 Mosaic forms of Mn-lipoxygenase

In order to improve the properties of Mn-lipoxygenase we plan substitute various parts with the corresponding sequence of soybean lipoxygenase using the α-helix information described above.

Example 7: Screening of eukaryotic DNA

To screen for homologous lipoxygenase genes in eukaryotic fungal strains, southern hybridization was performed on the genomic DNA from several fungal strains using cDNA of *Gaeumannomyces graminis* LOX gene as the probe. Strains of the following species were tested; *Pyricularia oryzae*, *Psaliota campestris*, *Penicillium roqueforti* and *Geotrichum candidum* ATCC34614. Genomic DNA was isolated as described in Example 2.

The probe was labeled with digoxigenin-dUTP using DIG DNA labeling Mix (Boehringer Mannheim) as follows; DIG labeled probe was prepared by PCR using primer 6 (SEQ ID NO: 14) and primer 7 (SEQ ID NO: 15) as the full-length cDNA of *G. graminis* LOX. PCR reaction mixture contained 0.1ug of pSG26 as the template, 1.25mM dNTP, 8% DIG DNA Labeling Mix, 30 pmol each of primer 6 and 7, 1 unit of LA taq polymerase (Takara) and GC buffer. Reaction conditions were as shown below. LA taq polymerase was added to the reaction mixture after step 1.

Step	Temperature	Time
1	98 °C	10 mins
2	94 °C	2 mins
3	60 °C	30 sec
4	72 °C	2 mins
5	72 °C	10 mins

* Step 2 to Step 4 were repeated 30 times.

PCR products were gel-purified and denatured by heating at 98 ºC before use.

About 5 micro-g of DNA digested with restriction enzyme was separated on 1.0% agarose gel and denatured by soaking the gel in 0.2N HCl for 30 minutes and in 0.5N NaOH + 1.5M NaCl for 30 minutes, then and neutralized in 1M Tris (pH 7.5) +1.5M NaCl for 30 minutes. Denatured DNA was then transferred to the nylon membrane by vacuum transfer with 20xSSC for 15 minutes. After fixing by UV irradiation, nylon membrane was used for the hybridization. Hybridization solution was composed with 5xSSC, 0.5% blocking reagent (Boehringer Mannheim), 0.1% N-lauroylsarcosine and 0.02% SDS. The nylon membrane was prehybridized with the hybridization solution at 60°C for 1 hour. After that, the heat-denatured DIG-labeled probe was added to the hybridization solution and incubated at 60°C overnight. Resulting membrane was washed with washing buffer comprising 2xSSC + 0.1%

SDS for 5 minutes at room temperature twice followed by washing with washing buffer 2 composed with 0.1xSSC + 0.1% SDS for 15 minutes at hybridization temperature twice. Washed membrane was air-dried and used for the detection of DIG-labeled DNA by following the provided protocol of DNA detection Kit (Boehringer Mannheim).

As the result, *Pyricularia oryzae* showed clear positive signals and *Geotrichum candidum* showed very weak signals. The results indicate that *Pyricularia oryzae* has a lipoxygenase gene that has a high identity to *Gaeumannomyces graminis* LOX and *Geotrichum candidum* has a gene that has low identity to *G. graminis* LOX.

Example 8: Effect of pH on Mn-lipoxygenase

The activity of lipoxygenase produced as in Example 4 was tested at various pH values. The enzyme was found to have a broad pH optimum with high activity in the range of pH 6-10 or 7-11 with linoleic acid or linolenic acid as substrate.

The stability of the enzyme was determined after 1hour incubation at 40°C at various pH values. The enzyme was found to have good stability in the pH range 4-10.

Example 9: Substrate specificity of lipoxygenase

The activity of lipoxygenase produced as in Example 4 was tested on various substrates as described above. The results are expressed as k_{cat} (or V_{max}), K_M and k_{cat}/K_M according to the Michaelis-Menten equation:

Substrate	k_{cat} micro-mol/min/mg	K_M mM	k_{cat}/K_M
Linoleic acid	5.63	0.0068	828
Arachidonic acid	0.296	0.0175	16.9
Linoleyl alcohol	3.32	0.0034	982
Methyl linoleate	1.37	0.164	8.39
Monolinolein			85.4
1,3-dilinolein			12.4
Trilinolein			9.15

The lipoxygenase showed about twice as high activity toward linolenic acid than linoleic acid at pH 7.

Example 10: Bleaching of β-carotene by native Mn-lipoxygenase

Purified Mn-lipoxygenase was used to bleach beta-carotene at pH 4.5, 6.5 and 9.5. The highest bleaching activity was found at pH 6.5.

Example 11: Effect of LAS on Mn-lipoxygenase

The activity of *G. graminis* lipoxygenase produced as in Example 4 was measured with LAS up to 400 ppm at pH 7.0 and pH10. The lipoxygenase was found to be fully stable against LAS up to 400 ppm (the highest concentration tested) at pH 7 and 10. This indicates that the lipoxygenase is stable enough at normal washing conditions, typically pH 10 with 200 ppm of LAS.

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.03.2001)
0-2	International Application No.	PCT/DK01/00574
0-3	Applicant's or agent's file reference	10072-WO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	3
1-2	line	7-9
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	05 July 2000 (05.07.2000)
1-3-4	Accession Number	DSMZ 13586
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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0-4	This form was received with the international application: (yes or no)	Yes
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CLAIMS

1. A polypeptide having lipoxygenase enzyme activity which:

a) has an amino acid sequence which has at least 50 % identity with the mature polypeptide of SEQ ID NO: 2 or 23;

b) is encoded by a nucleic acid sequence which hybridizes at 55°C with a complementary strand of the nucleic acid sequence encoding the mature polypeptide of SEQ ID NO: 1 or a subsequence thereof having at least 100 nucleotides;

c) has an amino acid sequence which can be obtained from the mature polypeptide of SEQ ID NO: 2 or 23 by substitution, deletion, and/or insertion of one or more amino acids; or

d) is encoded by the lipoxygenase-encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 13586.

2. A polynucleotide which comprises:

a) the partial DNA sequence encoding a mature lipoxygenase cloned into a plasmid present in *Escherichia coli* DSM 13586,

b) the partial DNA sequence encoding a mature lipoxygenase shown in SEQ ID NO: 2 or 23,

c) an analogue of the sequence defined in a) or b) which encodes a lipoxygenase and

i) has at least 50 % identity with said DNA sequence, or

ii) hybridizes at low stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

3. The polynucleotide of the preceding claim wherein the partial DNA sequence is the mature peptide-coding sequence shown in SEQ ID NO: 1 or 22.

4. A nucleic acid construct comprising the polynucleotide of claim 2 or 3 operably linked to one or more control sequences capable of directing the expression of the lipoxygenase in a suitable expression host.

5. A recombinant expression vector comprising the nucleic acid construct of claim 4, a promoter, and transcriptional and translational stop signals.

6. A recombinant host cell transformed with the nucleic acid construct of claim 4 or the vector of claim 5.

7. A method for producing a lipoxygenase comprising

a) cultivating the host cell of claim 6 under conditions conducive to production of the lipoxygenase, and

b) recovering the lipoxygenase.

8. An oligonucleotide probe which consists of at least 20 nucleotides and which encodes a partial polypeptide sequence of SEQ ID NO: 2 or 23.

9. A method for obtaining a polypeptide with lipoxygenase activity, comprising:

a) preparing a eukaryotic DNA library,

b) screening the library to select DNA molecules which hybridize to the probe of claim 8,

c) transforming host cells with the selected DNA molecules,

d) cultivating the transformed host cells to express polypeptides encoded by the DNA molecules, and

e) assaying the expressed polypeptides to select polypeptides having lipoxygenase activity.

10. A dough composition comprising a manganese lipoxygenase.

11. The composition of the preceding claim wherein the lipoxygenase is the polypeptide of claim 1.

12. A method for preparing a dough or a baked product made from dough, comprising adding a manganese lipoxygenase to the dough.

13. A method of oxygenating a substrate selected from the group consisting of linolenic acid, arachidonic acid, linoleyl alcohol and a linoleic acid ester comprising contacting the substrate in the presence of oxygen with a manganese lipoxygenase.

14. The method of the preceding claim wherein the ester of linoleic acid is methyl linoleate, monolinolein, dilinolein or trilinolein.

15. The method of any of claims 12-14 wherein the lipoxygenase is the polypeptide of claim 1.

16. A detergent composition comprising a manganese lipoxygenase and a surfactant.

17. The composition of the preceding claim wherein the surfactant comprises anionic surfactant, particularly linear alkyl benzenesulfonate.

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ctgagcgaca ggcacccggt catgggcgtg ctcaaccgcc tcatgtacca ggcctacgcc 480

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gggttccagg ccggctacct ggagaaggac ctgcggagcc gggggctggt cggcgaggac 660

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acgcacgtgg cctgggtcac gggcggggcg caccacgtca tgaaccaggg ctcgcccgtc 960

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tgaagagcga ggatggacat tggaggtatt gggtggtaat taacagcatg tggagggagg 1500

gctacacgag ccaaactctg taatggatgg ccaccagctg ctagtcagca gttcccacat 1560

tccccagaat cacggctacc gaatcgaatg ttcacagcaa aaaaaaaaaa a 1611

<210> 7

<211> 1857

<212> DNA

<213> Gaeumannomyces graminis

<400> 7

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gctgccgaag acgctgcggc gacgctgtct ttgacgtcca gcgcctccag caccaccgtg 120
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ccgttcgtct ggaccgcgct caatcccgcc gtcaaccctt ttttcctaag cgtctaa 1857

<210> 8

<211> 216

<212> PRT

<213> Gaeumannomyces graminis

<400> 8

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Arg Gly Gly Phe Lys Thr Leu Asp Asp Phe Ala Leu Leu Tyr Asn Gly
1               5                  10                  15

His Trp Lys Glu Ser Val Pro Glu Gly Ile Ser Lys Gly Met Leu Ser
            20                  25                  30

Asn Tyr Thr Ser Asp Leu Leu Phe Ser Met Glu Arg Leu Ser Ser Asn
        35                  40                  45

Pro Tyr Val Leu Lys Arg Leu His Pro Ala Lys Asp Lys Leu Pro Phe
    50                  55                  60

Ser Val Glu Ser Lys Val Val Lys Lys Leu Thr Ala Thr Thr Leu Glu
65                  70                  75                  80

Ala Leu His Lys Gly Gly Arg Leu Phe Leu Val Asp His Ser Tyr Gln
                85                  90                  95

Lys Lys Cys Thr Pro Gln Pro Gly Arg Tyr Ala Ala Ala Cys Gln Gly
            100                 105                 110

Leu Phe Tyr Leu Asp Ala Arg Ser Asn Gln Phe Leu Pro Leu Ala Ile
        115                 120                 125

Lys Thr Asn Val Gly Ala Asp Leu Thr Tyr Thr Pro Leu Asp Asp Lys
    130                 135                 140

Asn Asp Trp Leu Leu Ala Lys Ile Met Phe Asn Asn Asn Asp Leu Phe
145                 150                 155                 160

Tyr Ser Gln Met Tyr His Val Leu Phe His Thr Ile Pro Glu Ile Val
                165                 170                 175

His Glu Ala Ala Phe Arg Thr Leu Ser Asp Arg His Pro Val Met Gly
            180                 185                 190

Val Leu Asn Arg Leu Met Tyr Gln Ala Tyr Ala Ile Arg Pro Val Gly
        195                 200                 205

Gly Ala Val Leu Phe Asn Pro Gly
    210                 215
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<210> 9

<211> 15

<212> DNA

<213> Artificial

<220>

<221> misc_feature

<223> Primer 1

<220>

<221> modified_base

<222> (9)..(9)

<223> I

<220>

<221> misc_feature

<222> (9)..(9)

<223> I

<400> 9
gccctsccna acaac 15

<210> 10

<211> 23

<212> DNA

<213> Artificial

<220>

<221> misc_feature

<223> Primer 2

<400> 10
gcsggsaggc cgaagttctg gtc 23

<210> 11

<211> 29

<212> DNA

<213> Artificial

<220>

<221> modified_base

<222> (3)..(6)

<223> I

<220>

<221> misc_feature

<222> (3)..(6)

<223> I

<400> 11
ccncccngggt traasagsac sgcscccscc 29

<210> 12

<211> 36

<212> DNA

<213> Artificial

<220>

<221> misc_feature

<223> Primer 4

<400> 12
cggctgtcct ccaacccctta cgtcctcaag cgcctc 36

<210> 13

<211> 36

<212> DNA

<213> Artificial

<220>

<221> misc_feature

<223> Primer 5

<400> 13
gaggcgcttg aggacgtaag ggttggagga cagccg 36

<210> 14

<211> 36

<212> DNA

<213> Artificial

<220>

<221> misc_feature

<222> (4)..(9)

<223> Bgl II site

<220>

<221> misc_feature

<223> Primer 6

<400> 14
ggaagatcta tgcgctccag gatccttgcc atagtc 36

<210> 15

<211> 38

<212> DNA

<213> Artificial

<220>

<221> misc_feature

<222> (4)..(9)

<223> Xho I site

<220>

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<223> Primer 7

<400> 15
ccgctcgagt tagacgctta ggaaaaaagg gttgacgg 38

<210> 16

<211> 24

<212> PRT

<213> Gaeumannomyces graminis

<400> 16

Gly Leu Ser Gln Gly Met Pro Phe Val Trp Thr Ala Leu Asn Pro Ala
1 5 10 15

Val Asn Pro Phe Phe Leu Ser Val
20

<210> 17

<211> 27

<212> PRT

<213> Gaeumannomyces graminis

<400> 17

Gly Ala Thr Gly Asp Gly Thr Arg Ala Gly Leu Leu Ala Trp Leu Pro
1 5 10 15

Asp Glu Arg Gln Ala Val Glu Gln Val Ser Leu
20 25

<210> 18

<211> 22

<212> PRT

<213> Gaeumannomyces graminis

<400> 18

Gly Met Leu Ser Asp Tyr Thr Ser Asp Leu Leu Phe Ser Met Glu Arg
1 5 10 15

Leu Ser Ser Asn Pro Tyr
20

<210> 19

<211> 20

<212> PRT

<213> Gaeumannomyces graminis

<400> 19

Phe Ser Gly Val Leu Pro Leu His Pro Ala Ala Leu Tyr Ala Pro Ile
1 5 10 15

Ile Thr Thr Lys
20

<210> 20

<211> 25

<212> PRT

<213> Gaeumannomyces graminis

<220>

<221> misc_feature

<222> (17)..(17)

<223> Unknown

<400> 20

Ala Ile Arg Pro Val Gly Gly Ala Val Leu Phe Asn Pro Gly Gly Phe
1 5 10 15

Xaa Asp Gln Asn Phe Gly Leu Pro Ala
20 25

<210> 21

<211> 19

<212> PRT

<213> Gaeumannomyces graminis

<220>

<221> misc_feature

<222> (6)..(18)

<223> Unknown

<400> 21

Ala Leu Pro Asn Asn Xaa Pro Ala Ala Arg Thr Ala Lys Leu His Xaa
1 5 10 15

Leu Xaa Leu

<210> 22

<211> 1857

<212> DNA

<213> Gaeumannomyces graminis

<220>

<221> CDS

<222> (1)..(1854)

<223>

<220>

<221> mat_peptide

<222> (49)..()

<223>

<400> 22

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atg cgc tcc agg atc ctt gct ata gtc ttc gca gca cgc cat gtg gca       48
Met Arg Ser Arg Ile Leu Ala Ile Val Phe Ala Ala Arg His Val Ala
    -15                 -10                 -5                  -1

gcg ctg cca ctc gct gcc gaa gac gct gcg gcg acg ctg tct ttg acg       96
Ala Leu Pro Leu Ala Ala Glu Asp Ala Ala Ala Thr Leu Ser Leu Thr
1               5                   10                  15

tcc agc gcc tcc agc acc acc gtg ctc ccg tct ccg acc cag tac acg      144
Ser Ser Ala Ser Ser Thr Thr Val Leu Pro Ser Pro Thr Gln Tyr Thr
            20                  25                  30

ctg ccc aac aaa gac ccc aac cag ggg gca cgc aac gcc agt ata gcg      192
Leu Pro Asn Lys Asp Pro Asn Gln Gly Ala Arg Asn Ala Ser Ile Ala
        35                  40                  45

cgg aag cgg gag ttg ttc ctc tac ggc cca tcc acg ctc ggg cag acg      240
Arg Lys Arg Glu Leu Phe Leu Tyr Gly Pro Ser Thr Leu Gly Gln Thr
    50                  55                  60

acc ttc tac cct acc gga gag cta ggg aac aat atc tcg gcc cgc gac      288
Thr Phe Tyr Pro Thr Gly Glu Leu Gly Asn Asn Ile Ser Ala Arg Asp
65                  70                  75                  80

gtg ctg ctt tgg cgc caa gat gcg gcg aac cag acg gca acg gcg tac      336
Val Leu Leu Trp Arg Gln Asp Ala Ala Asn Gln Thr Ala Thr Ala Tyr
                85                  90                  95

cgc gaa gcc aat gag acg ttt gca gat att acc agc cgt ggc ggt ttc      384
Arg Glu Ala Asn Glu Thr Phe Ala Asp Ile Thr Ser Arg Gly Gly Phe
            100                 105                 110
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aaa acg ctc gac gac ttt gcg ctc ctc tac aat ggt cac tgg aag gag      432
Lys Thr Leu Asp Asp Phe Ala Leu Leu Tyr Asn Gly His Trp Lys Glu
        115                 120                 125

tcg gtt ccg gag ggc ata tcg aag ggc atg ttg agc aac tac acc tcg      480
Ser Val Pro Glu Gly Ile Ser Lys Gly Met Leu Ser Asn Tyr Thr Ser
    130                 135                 140

gac ctt ctc ttt tcc atg gag cgg ctg tcc tcc aac cct tac gtc ctc      528
Asp Leu Leu Phe Ser Met Glu Arg Leu Ser Ser Asn Pro Tyr Val Leu
145                 150                 155                 160

aag cgc ctc cac cca acc aag gac aaa ctg ccg ttc agc gtc gag agc      576
Lys Arg Leu His Pro Thr Lys Asp Lys Leu Pro Phe Ser Val Glu Ser
                165                 170                 175

aag gtg gtg aag aag ctg acg gcc acc acg ctt gag gcg ctc cac aag      624
Lys Val Val Lys Lys Leu Thr Ala Thr Thr Leu Glu Ala Leu His Lys
            180                 185                 190

ggc ggc cgc ctg ttc ctc gtg gac cac agc tac cag aag aag tac acc      672
Gly Gly Arg Leu Phe Leu Val Asp His Ser Tyr Gln Lys Lys Tyr Thr
        195                 200                 205

ccc cag cca gga cgg tac gcc gcg gcc tgc cag ggg ctt ttc tac ctg      720
Pro Gln Pro Gly Arg Tyr Ala Ala Ala Cys Gln Gly Leu Phe Tyr Leu
    210                 215                 220

gac gcg cgg tcc aac cag ttc ctg cct ctg gca atc aag acc aac gtg      768
Asp Ala Arg Ser Asn Gln Phe Leu Pro Leu Ala Ile Lys Thr Asn Val
225                 230                 235                 240

ggg gtg gat ctg acg tac acg ccc ctc gac gac aag gac gac tgg ctg      816
Gly Val Asp Leu Thr Tyr Thr Pro Leu Asp Asp Lys Asp Asp Trp Leu
                245                 250                 255

ctg gcc aag atc atg ttc aac aac aac gac ctg ttc tac tcc cag atg      864
Leu Ala Lys Ile Met Phe Asn Asn Asn Asp Leu Phe Tyr Ser Gln Met
            260                 265                 270

tac cac gtg ctc ttc cac acg atc ccc gag atc gtg cac gag gcc gcc      912
Tyr His Val Leu Phe His Thr Ile Pro Glu Ile Val His Glu Ala Ala
        275                 280                 285

ttc cgg acg ctg agc gac agg cac ccg gtc atg ggc gtg ctc aac cgc      960
Phe Arg Thr Leu Ser Asp Arg His Pro Val Met Gly Val Leu Asn Arg
    290                 295                 300

ctc atg tac cag gcc tac gcc atc cgg ccc gtg ggc ggg gct gtg ctc     1008
Leu Met Tyr Gln Ala Tyr Ala Ile Arg Pro Val Gly Gly Ala Val Leu
305                 310                 315                 320

ttc aac ccc ggc ggg ttc tgg gac caa aac ttt ggc ctg ccc gcc tcg     1056
Phe Asn Pro Gly Gly Phe Trp Asp Gln Asn Phe Gly Leu Pro Ala Ser
                325                 330                 335

gcc gcc atc gac ttc ccc ggc tcc gtg tac gcg cag ggc ggg ggc ggg     1104
Ala Ala Ile Asp Phe Pro Gly Ser Val Tyr Ala Gln Gly Gly Gly Gly
            340                 345                 350

ttc cag gcc ggc tac ctg gag aag gac ctg cgg agc cgg ggg ctg atc     1152
Phe Gln Ala Gly Tyr Leu Glu Lys Asp Leu Arg Ser Arg Gly Leu Ile
        355                 360                 365

ggc gag gac agc ggc ccg cgg ctg ccg cac ttc ccc ttc tac gag gac     1200
Gly Glu Asp Ser Gly Pro Arg Leu Pro His Phe Pro Phe Tyr Glu Asp
    370                 375                 380
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gcg cac cgc ctg atc ggg gcg atc cgg cgc ttc atg cag gcg ttc gtg      1248
Ala His Arg Leu Ile Gly Ala Ile Arg Arg Phe Met Gln Ala Phe Val
385                 390                 395                 400

gac tcg acg tac ggt gcc gac gac ggc gac gac ggg gcg ctg ctg cgc      1296
Asp Ser Thr Tyr Gly Ala Asp Asp Gly Asp Asp Gly Ala Leu Leu Arg
                405                 410                 415

gac tat gag cta cag aac tgg atc gcc gag gcc aac ggg ccg gcg cag      1344
Asp Tyr Glu Leu Gln Asn Trp Ile Ala Glu Ala Asn Gly Pro Ala Gln
            420                 425                 430

gtg cgc gac ttc ccc gcg gcg ccg ctg cga cgg cgc gcg cag ctg gtg      1392
Val Arg Asp Phe Pro Ala Ala Pro Leu Arg Arg Arg Ala Gln Leu Val
        435                 440                 445

gac gtg ctg acg cac gtg gcc tgg atc acg ggc ggg gcg cac cac gtc      1440
Asp Val Leu Thr His Val Ala Trp Ile Thr Gly Gly Ala His His Val
    450                 455                 460

atg aac cag ggc tcg ccc gtc aag ttc tcg ggg gtg ctg ccg ctg cac      1488
Met Asn Gln Gly Ser Pro Val Lys Phe Ser Gly Val Leu Pro Leu His
465                 470                 475                 480

ccg gcg gcg ctg tac gcg ccc atc ccg acg gcc aag ggc gcc acc ggc      1536
Pro Ala Ala Leu Tyr Ala Pro Ile Pro Thr Ala Lys Gly Ala Thr Gly
                485                 490                 495

aac ggg acg agg gcg ggc ctg ctg gcg tgg ctg ccc aac gag cgg cag      1584
Asn Gly Thr Arg Ala Gly Leu Leu Ala Trp Leu Pro Asn Glu Arg Gln
            500                 505                 510

gcc gtg gag cag gtc tcg ctg ctc gcg cgc ttc aac cgt gcc cag gtc      1632
Ala Val Glu Gln Val Ser Leu Leu Ala Arg Phe Asn Arg Ala Gln Val
        515                 520                 525

ggg gac agg aag cag acg gtg cgc gac gcc ttc gcc gcg ccc gac ctg      1680
Gly Asp Arg Lys Gln Thr Val Arg Asp Ala Phe Ala Ala Pro Asp Leu
    530                 535                 540

ctg gcc ggc aac ggg ccg ggg tac gcg gcg gcc aac gcg agg ttc gtc      1728
Leu Ala Gly Asn Gly Pro Gly Tyr Ala Ala Ala Asn Ala Arg Phe Val
545                 550                 555                 560

gag gac acg ggc cgt ata agt cgc gag att gcg ggc aga ggg ttt gac      1776
Glu Asp Thr Gly Arg Ile Ser Arg Glu Ile Ala Gly Arg Gly Phe Asp
                565                 570                 575

ggc aag ggc ctc agc cag ggc atg ccg ttc gtc tgg acc gcg ctc aat      1824
Gly Lys Gly Leu Ser Gln Gly Met Pro Phe Val Trp Thr Ala Leu Asn
            580                 585                 590

ccc gcc gtc aac cct ttt ttc ctg agc gtc taa                          1857
Pro Ala Val Asn Pro Phe Phe Leu Ser Val
        595                 600
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<210> 23

<211> 618

<212> PRT

<213> Gaeumannomyces graminis

<400> 23

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Met Arg Ser Arg Ile Leu Ala Ile Val Phe Ala Ala Arg His Val Ala
 -15                 -10                 -5                  -1

Ala Leu Pro Leu Ala Ala Glu Asp Ala Ala Ala Thr Leu Ser Leu Thr
1               5                   10                  15

Ser Ser Ala Ser Ser Thr Thr Val Leu Pro Ser Pro Thr Gln Tyr Thr
            20                  25                  30

Leu Pro Asn Lys Asp Pro Asn Gln Gly Ala Arg Asn Ala Ser Ile Ala
        35                  40                  45

Arg Lys Arg Glu Leu Phe Leu Tyr Gly Pro Ser Thr Leu Gly Gln Thr
    50                  55                  60

Thr Phe Tyr Pro Thr Gly Glu Leu Gly Asn Asn Ile Ser Ala Arg Asp
65                  70                  75                  80

Val Leu Leu Trp Arg Gln Asp Ala Ala Asn Gln Thr Ala Thr Ala Tyr
                85                  90                  95

Arg Glu Ala Asn Glu Thr Phe Ala Asp Ile Thr Ser Arg Gly Gly Phe
            100                 105                 110

Lys Thr Leu Asp Asp Phe Ala Leu Leu Tyr Asn Gly His Trp Lys Glu
        115                 120                 125

Ser Val Pro Glu Gly Ile Ser Lys Gly Met Leu Ser Asn Tyr Thr Ser
    130                 135                 140

Asp Leu Leu Phe Ser Met Glu Arg Leu Ser Ser Asn Pro Tyr Val Leu
145                 150                 155                 160

Lys Arg Leu His Pro Thr Lys Asp Lys Leu Pro Phe Ser Val Glu Ser
                165                 170                 175

Lys Val Val Lys Lys Leu Thr Ala Thr Thr Leu Glu Ala Leu His Lys
            180                 185                 190

Gly Gly Arg Leu Phe Leu Val Asp His Ser Tyr Gln Lys Lys Tyr Thr
        195                 200                 205

Pro Gln Pro Gly Arg Tyr Ala Ala Ala Cys Gln Gly Leu Phe Tyr Leu
    210                 215                 220

Asp Ala Arg Ser Asn Gln Phe Leu Pro Leu Ala Ile Lys Thr Asn Val
225                 230                 235                 240

Gly Val Asp Leu Thr Tyr Thr Pro Leu Asp Asp Lys Asp Asp Trp Leu
                245                 250                 255
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Leu Ala Lys Ile Met Phe Asn Asn Asn Asp Leu Phe Tyr Ser Gln Met
260 265 270

Tyr His Val Leu Phe His Thr Ile Pro Glu Ile Val His Glu Ala Ala
275 280 285

Phe Arg Thr Leu Ser Asp Arg His Pro Val Met Gly Val Leu Asn Arg
290 295 300

Leu Met Tyr Gln Ala Tyr Ala Ile Arg Pro Val Gly Gly Ala Val Leu
305 310 315 320

Phe Asn Pro Gly Gly Phe Trp Asp Gln Asn Phe Gly Leu Pro Ala Ser
325 330 335

Ala Ala Ile Asp Phe Pro Gly Ser Val Tyr Ala Gln Gly Gly Gly Gly
340 345 350

Phe Gln Ala Gly Tyr Leu Glu Lys Asp Leu Arg Ser Arg Gly Leu Ile
355 360 365

Gly Glu Asp Ser Gly Pro Arg Leu Pro His Phe Pro Phe Tyr Glu Asp
370 375 380

Ala His Arg Leu Ile Gly Ala Ile Arg Arg Phe Met Gln Ala Phe Val
385 390 395 400

Asp Ser Thr Tyr Gly Ala Asp Asp Gly Asp Asp Gly Ala Leu Leu Arg
405 410 415

Asp Tyr Glu Leu Gln Asn Trp Ile Ala Glu Ala Asn Gly Pro Ala Gln
420 425 430

Val Arg Asp Phe Pro Ala Ala Pro Leu Arg Arg Arg Ala Gln Leu Val
435 440 445

Asp Val Leu Thr His Val Ala Trp Ile Thr Gly Gly Ala His His Val
450 455 460

Met Asn Gln Gly Ser Pro Val Lys Phe Ser Gly Val Leu Pro Leu His
465 470 475 480

Pro Ala Ala Leu Tyr Ala Pro Ile Pro Thr Ala Lys Gly Ala Thr Gly
485 490 495

Asn Gly Thr Arg Ala Gly Leu Leu Ala Trp Leu Pro Asn Glu Arg Gln
500 505 510

Ala Val Glu Gln Val Ser Leu Leu Ala Arg Phe Asn Arg Ala Gln Val
515 520 525

Gly Asp Arg Lys Gln Thr Val Arg Asp Ala Phe Ala Ala Pro Asp Leu
530 535 540

Leu Ala Gly Asn Gly Pro Gly Tyr Ala Ala Ala Asn Ala Arg Phe Val
545 550 555 560

Glu Asp Thr Gly Arg Ile Ser Arg Glu Ile Ala Gly Arg Gly Phe Asp
565 570 575

Gly Lys Gly Leu Ser Gln Gly Met Pro Phe Val Trp Thr Ala Leu Asn
580 585 590

Pro Ala Val Asn Pro Phe Phe Leu Ser Val
595 600

<210> 24

<211> 133

<212> DNA

<213> Gaeumannomyces graminis

<400> 24
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tttctttcgc acttggtatt ggtacctacc tacccaccta acctaggtgc taacacgtct 120

cgttgggcta tag 133